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Research Article
Bactericide, Antioxidant and Cytotoxic Activities from Marine Algae of Genus *Laurencia* Collected in Baja California Sur, México

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Abstract

Background and Objective: Marine environment represents countless and diverse resource for new drugs to combat major diseases. Extracts from four *Laurencia* species (*L. johnstonii, L. pacifica, L. gardneri* and *L. papillosa)* from Baja California Sur, México were evaluated for their antioxidant, antibacterial and cytotoxic activity. **Methodology:** The antioxidants activity of *Laurencia* sp. were evaluated using the radical scavenging activity in three *in vitro* radicals: 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and nitric oxide (NO). The antibacterial activity was evaluated by the broth microdilution method to determinate the Minimum Inhibitory Concentrations (MIC) against *Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis, Micrococcus luteus, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The cytotoxicity was analyzed on HeLa (cervix adenocarcinoma) and Vero (kidney epithelial) cells, using the reduction of tetrazolium salt WST-1. **Results:** The seaweed of genus *Laurencia* demonstrated an overall low activity, with half maximal effective concentration (EC_{50}) values >1.5 mg mL^{-1}. *Laurencia pacifica* showed the best biocide effects with MIC of 6.25 μg mL^{-1} against Gram positive bacterial and cytotoxic potential with half inhibitory concentration (IC_{50}) <30 μg mL^{-1} against Vero and HeLa cells. **Conclusion:** Some *Laurencia* species have a great antibacterial and cytotoxic activity which could be considered for future studies.

Key words: *Laurencia*, seaweeds, algal extract, marine antibacterial, antioxidant activity, cytotoxic activity, bioactive metabolites, bioprospection

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

About 70% of the earth's surface is covered with water and it comprises 500,000 live species divided into 30 different phyla, holding an outstanding potential for discovery and development of bioactive natural products[1]. The search for new metabolites from marine organisms has resulted in the isolation over 10,000 such compounds[2], many of which are endowed with pharmacodynamic properties[3].

There is an ongoing gold rush among pharmaceutical and biotechnology companies to tap this new found resource[1] and among the myriad of marine organisms, algae are one of the richest and most promising sources of bioactive primary and secondary metabolites[4]. They synthesize a variety of compounds such as carotenoids, amino acids, acetogenins, antioxidants such polyphenols, alkaloids, halogenated compounds and polysaccharides such as agar, carrageenan, proteoglycans, alginate, laminaran, rhamnan sulfate, galactosyl glycerol and fucoidan[5]. Seaweeds are known to be rich source of important bioactive compounds with a range of effects as anticancer, antiparasitic, antibacterial and insecticidal properties[6].

Red algae of the genus *Laurencia* which are distributed throughout the world and are most abundant in tropical and subtropical waters, are highly prolific organisms. This genus is represented by approximately 80 species. Many of which have been proven to be some of the most important producers of structurally unusual secondary metabolites in the marine environment. To date, there are around 600 publications related to the chemistry of secondary metabolites and more than 1,100 different metabolites from this genus, many of which are C15-acetogenins, halogenated diterpenes and sesquiterpenes[7]. Due to their relatively high degree of halogenation, many of these molecules either are biologically active or play an active role in their ecosystem, often exhibiting antibacterial, antifungal, antiviral, anti-inflammatory, antiproliferative, cytotoxic, antifouling, antifeedant, ichthyotoxic and/or insecticidal activity[8,9]. Taking into account that natural marine products in recent years, they have been important source of new compounds with possible application in the pharmaceutical industry, the present document reports the bactericide, antioxidant and cytotoxic activities from marine algae of genus *Laurencia* collected in Baja California Sur, México. In this way, opening the possibility for the biopharmaceutical exploitation of this marine resource.

MATERIALS AND METHODS

Plant material and extract preparation: Algal samples were collected by hand in June-July 2015, on the coast from Baja California Sur, México. The samples were rinsed with tap water to remove salts, sand and epiphytes and sun-dried. The dried material was crushed using a blender and stored at -80°C. Samples were identified to genus and species level by Dr. Rafael Riosmena Rodríguez using taxonomic keys for future reference. The crushed material was soaked in ethanol for 3 days at 25°C under gentle agitation. Then, ethanol was filtered and replaced (3×1200 mL). Extracts were filtered through Whatman No. 4 filter paper and solvent removed using rotatory vacuum evaporator to yield dry material.

Antioxidant assays: DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity was determined using 100 µL extract (from 5-300 µg mL^{-1} in serial dilution) and 100 µL DPPH (0.12 mg mL^{-1}) in 96 well-microplate. The reaction was complete after 30 min in the dark at room temperature and the absorbance was read at 517 nm, noting a color to turn from purple to yellow[10]. Positive and negative controls were ascorbic acid (50-3.14 µg mL^{-1}) and methanol, respectively.

Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) assay was based on the ability of different substances to scavenge ABTS radical. The radical cation was prepared by mixing 10 mM ABTS stock solution with 2.45 mM potassium persulfate and leaving the mixture for 16 h. The ABTS solution was diluted with ethanol to absorbance of 0.7±0.05 at 734 nm. The photometric assay was conducted on 200 µL of ABTS solution and 20 µL of tested samples (0.25-2 mg mL^{-1}), measurements were taken at 734 nm after 6 min in dark[9]. Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was the control.

Nitric oxide (NO) scavenging activity can evaluated using the Griess reaction, the sodium nitroprusside is decompose in aqueous solution at physiological pH (7.2) producing NO. For the experiment two controls are necessary: NO production (PBS) and NO inhibition (ascorbic acid). Fifty microliter of sodium nitroprusside (5 mM) in phosphate buffered saline (PBS) was mixed with 50 µL of different concentrations of extract and incubated at room temperature for 2 h. After the incubation period, 100 µL of Griess reagent (1:1 1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added and the absorbance was read at 550 nm.

Antibacterial activity: The Minimum Inhibitory Concentration (MIC) was determinate in 96 well microplates by the broth microdilution method according to Santos *et al.*[11] with some modifications. The extracts were tested against Gram positive (*Staphylococcus aureus, Bacillus subtilis*, *Enterococcus faecalis* and *Micrococcus luteus*) and Gram negative bacteria (*Pseudomonas aeruginosa* and *Klebsiella*

pneumoniae). Samples were dissolved in Mueller-Hinton medium (10% DMSO) and 100 µL were added per well, madding serial dilutions of each extract (100-0.78 µg mL^{-1}) and positive control (16-0.125 µg mL^{-1}). The modification is to adapt the inoculum in 10 µL of inoculum containing 1.5×10^4 CFU colony-forming unit per mL (CFU mL^{-1}) of each microorganism was added to each well. Plates were incubated for 24 h at 37°C and 10 µL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were added to each well to detect the active bacterial metabolism, resulting in a purple color due to the insoluble formazan produced. Controls were medium (sterility control), inoculum (viability control) and gentamicin (positive control).

Cytotoxic assay: The cancer cell line HeLa (human cervix adenocarcinoma, ATCC- No. CCL-2) and normal cell line Vero (kidney epithelial monkey, ATCC-CCL81) were grown in minimal essential medium (MEM) with 10% fetal bovine, penicillin (100 U mL^{-1}), streptomycin (100 µg mL^{-1}) and incubated in an atmosphere of 5% CO_2 at 37°C. Cytotoxic assays were performed in 96 well microplates containing 5×10^3 cells/well. Cell cultures were exposed to different concentrations of samples for 24 h. Cell viability was measured by WST-1 assay after treatment, to do this, 10 µL of WST-1 were added to each well and after 90 min of incubation and the absorbance was measured at 450 nm. The concentration of the samples that inhibited 50% of cell growth (IC_{50}) was calculated from the log-dose inhibition growth curve obtained by a nonlinear regression algorithm. Taxol was used as positive control and cell culture medium and 1% DMSO were used as negative controls[12]. The results are presented as the Mean±SD.

Statistical analysis: The half maximum effective concentration (EC_{50}) and the half maximum inhibitory concentration (IC_{50}) were determinate using a probit regression, all experiments were performed in triplicate and the data are expressed as mean±standard deviation (SD). The values were analyzed using the SPSS version 17.0 software.

RESULTS

The antioxidant activity was evaluated by DPPH, ABTS and NO assays and the results are summarized in Table 1. The extracts were capable of scavenging radicals in a concentration-dependent manner; for DPPH radical the estimated median effective concentration (EC_{50}) values were between 1.39 and 1.69 mg mL^{-1} for *L. johnstonii*, *L. pacifica* and *L. papillosa* extracts, for ABTS radical *L. pacifica* and *L. papillosa* showed EC_{50} values of 3.77 and 3.39 mg mL^{-1}, respectively. The *L. gardneri* extract doesn't showed scavenging property at proved concentrations. For nitric oxide free radical scavenging activity, all of the four extracts showed EC_{50} between 3 and 4.76 mg mL^{-1}. Compared to Trolox, there is a significant difference between the antioxidant activities of the *Laurencia* sp. extracts.

The antibacterial activity of crude ethanol extracts of the 4 species of *Laurencia* algae genus was analyzed by microdilution method to determine their Minimum Inhibitory Concentration (MIC) against Gram positive and Gram negative bacteria and the results are summarized in Table 2. It was observed that the extract that showed the greatest biocidal effect was *L. pacifica*, with MIC of 6.25 µg mL^{-1} against all gram-positive bacteria tested; *L. gardneri* did not show inhibitory effect at the analyzed doses.

Table 1: Antioxidant activities of *Laurencia* sp. collected in B.C.S-México

Radical	*L. johnstonii*	*L. pacifica*	*L. papillosa*	*L. gardneri*	Control
*DPPH	1.53±0.08	1.69±0.09	1.39±0.08	>5	0.028±0.014
*ABTS	>5	3.77±0.10	3.39±0.10	>5	0.045±1.600
**NO	3.00±0.3	3.52±0.40	4.76±0.40	3.72±0.3	0.052±1.000

EC_{50}: Median effective concentration, DPPH: 2,2-Diphenyl-1-picrylhydrazyl, ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), NO: Nitric oxide, *Ctrl: Trolox, **Ctrl: Ascorbic acid. n = 3, ±standard deviation (SD)

Table 2: Minimum Inhibitory Concentration of *Laurencia* sp. collected in B.C.S-México

Bacteria	*L. johnstonii*	*L. pacifica*	*L. papillosa*	*L. gardneri*	Control*
Enterococcus faecalis	12.5	6.25	12.5	>100	2.0
Bacillus cereus	12.5	6.25	12.5	>100	1.0
Staphylococcus aureus	6.25	6.25	12.5	>100	0.5
Micrococcus luteus	6.25	6.25	12.5	>100	0.5
Pseudomonas aeruginosa	>100	>100	>100	>100	4.0
Klebsiella pneumoniae	>100	>100	>100	>100	4.0

*Gentamicin was used like a positive control. Data reported in µg mL^{-1}, n = 3, ±standard deviation (SD)

Table 3: Cytotoxic activity of *Laurencia* sp. collected in B.C.S-México

Cellular line	*L. johnstonii*	*L. pacifica*	*L. gardneri*	*L. papillosa*	Control
Vero	31.21±0.38	25.25±0.38	>50	29.25±0.33	19.20±0.08
HeLa	32.49±0.35	27.53±0.35	>50	21.81±0.36	15.66±0.01

*Taxol was used as positive control. Data reported in μg mL^{-1}, n = 3, ±standard deviation (SD)

The cytotoxicity of *Laurencia* sp. extracts after 24 h is showed in Table 3. The cytotoxic effect was estimated from the cell counts of the control cultures versus the cell populations after treatments. All the extracts, except *L. gardneri* showed IC_{50} values between 25.25 and 31.21 μg mL^{-1} against Vero and between 21.81 and 32.49 μg mL^{-1} for HeLa. The positive control (taxol) was more active than the extracts but it is a pure compound meanwhile the extracts are a mixture of compounds.

DISCUSSION

As a consequence of an increasing demand in screening for new therapeutic drugs from natural products, there is a greater interest towards marine organisms[13]. Development of the resistant bacteria is globally considered as major medical problem, thus leads to big threat for human society[14], being indispensable the search of new class of antibacterial substances. To date, it is known that red and brown algae are better source of antibacterial compound in comparison to green algae[4,15] and there are several reports about *Laurencia* extracts against Gram positive and Gram negative bacteria with MIC values between 0.5 and 500 μg mL^{-1}[16,16-18] and in some cases more than 5 mg mL^{-1}[19], These differences can be due to the species, the reproductive state, the season and geographical location of sampling or to the solvent used to extract[19,20]. In relation to the results obtained in this study, it was observed that the species of *Laurencia* analyzed only inhibited the Gram negative bacteria, the resistant mechanism of Gram negative bacteria is due to the permeability provided by the cell wall or to the membrane accumulation tactics[21]. Natural antioxidants found in many algae, are important bioactive compounds that play an important role against various diseases and ageing processes[22]. This necessity of new natural antioxidants has resulting in several reports using different assays due to various mechanisms of antioxidant action, as no single assay will accurately reflect all the radical sources or all antioxidants in a mixed or complex system[23]. The species analyzed in this study showed a weak antioxidant effect, in relation with this, Tariq *et al.*[24] evaluated the ethanol and water extracts of 15 seaweeds for their antioxidant potential by ABTS assay at 1 and 5 mg mL^{-1} and all of them showed EC_{50} values higher than 5 mg mL^{-1} for ethanolic extracts an higher than 1 mg mL^{-1} for water extracts. Nevertheless, Indu and Seenivasan *et al.*[25] showed EC_{50} values in DPPH, ABTS and NO lower than 1 mg mL^{-1} for three brown seaweed extracts. These results are in agreement with Kelman *et al.*[26], who determined the total antioxidant activity of organic extracts of 37 algal samples, comprising 27 different genera and they showed that the antioxidant activity varied significantly among algae and brown algae had the highest mean antioxidant, followed by the green algae and the red algae with the lowest activity. Zubia *et al.*[22] also evaluated the antioxidant potential of 48 species of macroalgae (17 Chlorophyta, 8 Phaeophyta and 23 Rhodophyta) from the coasts of Yucatán and Quintana Roo (México) and all the Rhodophyta showed antioxidant activities with EC_{50} values more than 2 mg mL^{-1}. Although the genus *Laurencia* represent a prolific source of secondary metabolites, their antioxidant activity was not comparable to other species from the order Ceramiales. There are several reports supporting the highest antioxidant activity of brown algae, due to their higher amounts of polyphenols[27-32].

Algal metabolites have isolated from species of the genus *Laurencia* have exhibited a wide spectrum of activities, with the majority of them reported to display cytotoxic and antibiotic properties[33,34].

Stein *et al.*[35] described a comparative study of the cytotoxic properties of extracts obtained from the *Laurencia* complex against a primary tumor cell line (MES-SA) and their results demonstrated highest levels of cytotoxicity for the non-polar (hexane) extracts and lowest levels with the polar (methanol) extracts, with IC_{50} until 5-fold higher for extracts of *Laurencia translucida.*

There are several reports of cytotoxic activity from metabolites isolated from *Laurencia* species, reporting IC_{50} values from 0.05 μM to more than 300 μM[8,36-40].

Results suggests a general cell toxicity, however, taking into account that the variability in the chemical composition of the extract depending on species it comes from, place and time of collection and the solvent used for the extraction, it would be desirable to continue studying the chemical and biological activities of said extracts.

CONCLUSION

The ethanol extract obtained from *Laurencia pacifica* showed the best biocide effect, presenting a natural

alternative for antibiotic against Gram positive bacteria and the tested cell lines. It is important to know the metabolites from these algae, identify the bioactive ones and determinate the structural-bioactivity relationship.

SIGNIFICANCE STATEMENT

The present study reports on the antioxidant, bactericide and cytotoxic activities of extracts from *Laurencia* specimens sampled from Baja California Sur, Mexico. It aims to expand upon the already existing knowledge regarding this genus bioactive profile, concurrently proposing the metabolic prospecting and further characterization of candidate compounds for pharmacological uses. Additionally, it provides a preliminary estimate of *Laurencia's* regional exploitation opportunities as a source of commercial natural products, given that bioactive compound production and make-up varies to the environmental conditions and genetic variables.

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